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GDF5 reduces MMP13 expression in human chondrocytes via DKK1 mediated canonical Wnt signaling inhibition

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SUMMARY

Objective: Growth differentiation factor 5 (GDF5) is important for joint formation and associated with osteoarthritis (OA). Its role for the homeostasis of cartilage extracellular matrix (ECM) is, however, unknown. The canonical Wnt signaling pathway is also implemented in OA and activation of the pathway has detrimental effects on the cartilage ECM. The objective of this study was to investigate the effect of GDF5 stimulation on the Wnt signaling pathway and on the expression of known modulators of cartilage ECM.

Design: Human chondrocytes were cultured in the pellet mass system and stimulated with increasing concentrations of GDF5. Expression of matrix modulating enzymes and canonical Wnt inhibitors dickkopf 1 (*DKK1*) and frizzled related protein (*FRZB*) were measured with quantitative PCR (qPCR). Protein levels of matrix metalloprotease 13 (MMP13), DKK1 and β-catenin were measured with enzyme-linked immunosorbent assay (ELISA). Canonical Wnt signaling was stimulated with Wnt3a and small molecule CHIR-99021 and DKK1 was blocked with small molecule WAY-262611.

Results: In this study, we show that GDF5 stimulation of human chondrocytes inhibits expression of the cartilage ECM degrading enzymes *MMP13* and *ADAMTS4* and stimulates the expression of cartilage anabolic genes *ACAN* and *SOX9*. We further show that the stimulation inhibits the canonical Wnt signaling pathway through expression of the canonical Wnt inhibitors DKK1 and FRZB. Finally we show that inhibition of MMP13 expression through GDF5 stimulation is mediated by DKK1.

Conclusion: Herein, we provide evidence of a previously unknown link between GDF5 signaling and canonical Wnt signaling that may contribute to the understanding of the molecular mechanisms of OA.

Introduction

Osteoarthritis (OA) is a progressive multifactorial disease where age, obesity and inheritance are predisposing factors, however the underlying molecular mechanisms are largely unknown[1,2].

The progression of the disease is a result of mechanical wear and an imbalance between anabolic and catabolic factors, including dysregulation of the main cartilage extracellular matrix (ECM) degrading enzymes matrix metalloprotease 13 (MMP13) and aggrecanases (ADAMTS4 and 5)[3,4]. A critical regulatory system for cartilage maintenance is the β-catenin mediated canonical Wnt signaling pathway. This pathway is important during cartilage development and its activation in the adult cartilage tissue leads to hypertrophy, initiation of calcification and tissue degradation via increased expression of ECM degrading components[5–8]. Wnt ligands show increased expressions after cartilage damage and in tissues affected by OA, resulting in the subsequent activation of the Wnt pathways in the cells[9–11].

The canonical Wnt signaling pathway ligands, such as Wnt3a, signal through the interaction with two types of receptors at the cell surface, the serpentine seven transmembrane Frizzled (FZD) family receptors and the single-pass transmembrane low density lipoprotein receptor related proteins 5 and 6 (LRP5/6). In the absence of Wnt ligands, glycogen synthase kinase 3β (GSK3β) phosphorylates the canonical Wnt signal mediator β-catenin, in a destruction complex with axin, casein kinase 1-α and adenomatous polyposis coli[2]. This phosphorylation leads to the ubiquitin-mediated degradation of β-catenin in the proteasomes. Activation of the canonical

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Wnt pathway takes place when the Wnt ligands bind to the receptors, forming a functional ligand–receptor complex. This will reduce the activity of GSK3β, resulting in an accumulation of newly synthesized β-catenin in the cell cytosol, which leads to the subsequent translocation of the protein into the cell nucleus[5,12,13]. Nuclear β-catenin interacts with DNA bound T-cell factor/lymphoid enhancer factor (TCF/LEF) proteins and thereby induces expression of Wnt target genes such as peroxisome proliferator-activated receptor delta (PPARD) and FOS-like antigen 1 (FOSL1)[5,13,14].

Important regulators of the canonical Wnt signaling pathway are the secreted Wnt antagonists frizzled related protein (FRZB) and dickkopf 1 (DKK1)[13]. FRZB binds to extracellular Wnt ligands, preventing them from interacting with the FZD receptors. DKK1 functions as an inhibitory ligand to the LRP5/6 receptor, thereby preventing the binding of the Wnt ligands to the receptor[13]. Single nucleotide polymorphisms (SNPs) in the FRZB gene have shown association with increased OA susceptibility[15,16], and dysregulation of the DKK1 expression is associated with increased OA progression[17–21].

Growth and differentiation factor 5 (GDF5) belongs to the bone morphogenetic protein (BMP) family and the transforming growth factor ß (TGFß) superfamily. Also known as BMP14 and cartilage derived matrix protein 1 (CDMP1), it was originally characterized as a protein from cartilage extracts that could induce cartilage and bone formation in subcutaneous implants[22–25]. It is a key regulator of mesenchyme condensation and chondrogenic differentiation in developing joints and mutations in the gene can lead to defects in the developing skeleton[22]. Mutations in the GDF5 gene have also been shown to cause increased susceptibility to develop OA. A single nucleotide polymorphism (SNP, rs143383 T/C) located in the 5′-UTR of the GDF5 gene has been shown to have a connection to hip and knee OA in a range of ethnic groups. The result of this mutation is a slight reduction in the activity of the GDF5 promoter, indicating that even a minor imbalance in the GDF5 expression can lead to OA[26,27]. What role GDF5 plays in the etiology of OA and how GDF5 affects other signaling pathways in human cartilage is however still unknown.

The aim of this paper was to investigate the effect of GDF5 signaling on the expression of human cartilage ECM modulating proteins. Furthermore we wanted to examine if GDF5 signaling affected the expression of Wnt inhibitors in human chondrocytes, and if that in turn had an impact on canonical Wnt signaling.

Methods

Cell expansion

Surplus human chondrocytes from five patients (average age 31) undertaking ACI were cultured in chondrocyte expansion medium consisting of Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, Invitrogen, Paisley, UK) supplemented with 0.1 mg/mL L-ascorbic acid (Apotekets produktionsenhet, Umeå, Sweden), 1× Penicillin–Streptomycin (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine (Invitrogen) and 10% human serum, at 37°C in 7% CO_2 and 90% relative humidity. Medium was changed two to three times per week. Subculture was made with trypsin-EDTA solution (0.125% trypsin (Invitrogen) diluted in 0.1 M PBS with 0.2 g/L EDTA) at 80% cell confluence. During expansion the cells undergo dedifferentiation, which is partially reverted during the following high-density pellet culture. The cells are in this study denoted as chondrocytes, from their original phenotype, independent of degree of differentiation.

Reference biopsy material

Cartilage biopsy reference material from five OA patients (Ahlbäck score > 2, Mankin score > 4.5) and six control donors (Mankin score < 1) was earlier characterized by Thorfve *et al.*[10] and mRNA was kindly provided for this study. Genes analyzed in the present study have not been studied in this material previously. The analysis performed in the present study is in accordance with the ethical approval of Thorfve *et al.* The expression levels of the analyzed genes were compared to unstimulated and 200 ng/ml GDF5 stimulated chondrocyte pellet cultures ($n = 3$).

Table I
The factorial design for the stimulation of pellets with GDF5 (−) 2 ng/ml, (c) 20 ng/ml, (+) 200 ng/ml and WAY-262601 (−) 0.01 μM, (c) 0.1 μM, (+) 1 μM

Nr	GDF5	WAY-262611
1	−	−
2	+	−
3	−	+
4	+	+
5	−	c
6	+	c
7	c	−
8	c	+
9	c	c
10	c	c
11	c	c

High-density pellet culture

Chondrocytes (passage 3) were resuspended at 1.25×10^6 cells/ml in basic differentiation media (BDM) consisting of DMEM high glucose (PAA Laboratories) supplemented with 100 nM dexamethasone (Sigma, St Louis, MO, USA), 1× insulin-transferrin-selenous acid premix (ITS) (Invitrogen), 1.0 mg/ml human serum albumin (HSA, Equitech-Bio, Kerrville, TX, USA) supplemented with 5.0 μg/ml linoleic acid (Sigma), 14 μg/ml L-ascorbic acid 2-phosphate (Sigma) and 1× Penicillin–Streptomycin. The cell suspension was divided into aliquots of 200 μl to each well of an uncoated flat bottom 96-well plate (Costar, Corning, NY, USA). The plate was centrifuged at 1,500 rpm for 5 min and incubated for 48 h at 37°C in 5% CO_2 and 90% relative humidity to allow for pellet formation. After 48 h the culture medium was changed to BDM supplemented with appropriate growth factors (GFs); 2, 10, 100 or 200 ng/ml recombinant GDF5 (Nordic biosite, Stockholm, Sweden) (dosage determined by in house pilot studies and previous published studies[28,29]), 50 ng/ml recombinant Wnt3a[7] (R&D systems, Minneapolis, MN, USA), 0.5 μM GSK3β inhibitor CHIR-99021 (Axon Medchem, Groningen, the Netherlands), 0.01, 0.1 or 1 μM DKK1 inhibitor WAY-262611 (Millipore, Billerica, MA, USA). Medium was changed three times per week for 21 days, whereafter the pellets were snap-frozen in liquid nitrogen and stored at −80°C.

Response surface modeling

Factorial design of experiments (DoE) was used for investigating co-stimulation of chondrocytes with GDF5 and WAY-262611 to address how these factors simultaneously affect the cells. In short, the DoE method allows for a systematic approach to optimization processes and for studying synergistic effects. It is based on defining mathematical relationships between input factors and output response. The results are often displayed in response surfaces showing which levels of input factors that result in the highest or lowest effect on the output variable. For a comprehensive review, please read Mandenius *et al.*[30]. The DoE setup for GDF5 and WAY-262611 interactions was designed with computer software Modde 8.0 (Umetrics, Umea, Sweden). This resulted in 11 different medium mixtures with combinations of low (−), mid (c), or high (+) concentration of the additives. For concentration combinations

see Table I. The 11 medium formulations were used to stimulate two chondrocyte pellets each. Pellets were cultured for 21 days in respective medium with media changes three times per week, whereafter the pellets were snap-frozen in liquid nitrogen and stored at −80°C.

Isolation of total RNA

Frozen pellet cultures were homogenized in 1.5 ml polypropylene tubes using stainless steel beads and a TissueLyser (Qiagen, Hilden, Germany) for 2 min at 25 Hz. QIAZol (Qiagen) was added to the samples and mixed for 2 min at 25 Hz. Chloroform was added (0.2 mL/mL QIAZol) and mixed. Tubes were centrifuged at 13,000 rpm for 15 min at 4°C. Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturers protocol. DNaseI was used to remove contaminating genomic DNA from the isolated RNA (Qiagen).

Quantitative PCR (qPCR)

All software and reagents for the analyses were purchased from Applied Biosystems (Carlsbad, CA, USA). Complementary deoxyribonucleic acid (cDNA) was prepared from 200 ng total RNA using the High Capacity cDNA Reverse Transcription Kit. qPCR was performed in duplicates with cDNA corresponding to 2.5 ng RNA and TaqMan Universal master mixture with 1× assay-on-demand mixes of primers for the genes (assay numbers in brackets): *ACAN* (Hs00153936_ml), *SOX9* (Hs00165814_m1), *COL10A1* (Hs00166657_m1), *COL2A1* (Hs_00156568), *GDF5* (Hs00167060_m1), *MMP13* (Hs00233992_m1), *ADAMTS4* (Hs00192708_m1), *TIMP1* (Hs99999139_m1), *TIMP3* (Hs00165949_m1), *DKK1* (Hs00183740_m1), *FRZB* (Hs00173503_m1), *PPARD* (Hs00602622_m1), *FOSL1* (Hs04187685_m1), with *PPIA* (Hs99999904_m1) as reference gene. PCR was performed using the 7900HT fast real-time PCR system (Life Technologies). Relative quantification of the target gene expression was performed according to the standard curve method calculated by the ddCq method with detection limit at 37 cycles for all genes except *COL10A1* where detection limit was set to 40 to allow for statistical analysis.

Size assessment of pellets

Pellets in the 96-well plates were investigated under the DIAPHOT300 microscope (Nikon, Corp., Tokyo, Japan) and photographed with a DXM1200 camera (Nikon). The size of the pellet was determined with the ImageJ software (National Institutes of Health, USA).

Biochemical analysis of pellets

On day 21 the pellets were digested with papain (Sigma) solution as previously described[31], mechanically dissolved by vortex and analyzed for DNA and glycosaminoglycan (GAG) content. Quantification of DNA was performed with a Hoechst 33258 (Sigma) assay, according to the manufacturers instructions. DNA values were normalized against unstimulated control for statistical analysis. Starting amounts of DNA at day 0 of pellet culture were estimated for 250,000 cells in each pellet. Per pellet, 250,000 cells gives 250,000 * 7.7 pg DNA/cell[32] = 1.9 μg DNA per pellet. GAG was quantified with a dimethylmethylene blue assay as previously described[33].

Histology of pellets

On day 21 the pellets were fixed in Histofix™ (Histolab Products AB, Gothenburg, Sweden), dehydrated and embedded in paraffin.

Table II
Effects on gene expression of ECM catabolic enzymes in human articular chondrocytes after GDF5 stimulation. For *MMP13* and *ADAMTS4* gene expression were reduced with increased GDF5 concentration. For their inhibitors, *TIMP1* expression was unaffected and *TIMP3* expression was increased in a GDF5 dose dependent manner ($n = 5$, three replicates). Results analyzed with ANOVA, table showing mean ± 95% CI

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5		*P*-value
MMP13							
No GF	1.00 ± 0.71	1.00 ± 0.00	1.00 ± 0.02	1.00 ± 0.54	1.00 ± 0.23		
GDF5 10 ng/ml	0.55 ± 0.31	0.91 ± 0.11	0.58 ± 0.14	0.24 ± 0.11	0.94 ± 0.30		0.93
GDF5 100 ng/ml	0.18 ± 0.07	0.17 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.50 ± 0.00	↓	*0.047*
GDF5 200 ng/ml	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.12 ± 0.01	↓	*0.00038*
ADAMTS4							
No GF	1.00 ± 0.21	1.00 ± 0.23	1.00 ± 0.26	1.00 ± 0.25	1.00 ± 0.35		
GDF5 10 ng/ml	0.99 ± 0.63	0.94 ± 0.12	0.48 ± 0.17	0.82 ± 0.43	1.05 ± 0.33		*0.73*
GDF5 100 ng/ml	0.75 ± 0.14	0.65 ± 0.03	0.32 ± 0.13	0.72 ± 0.05	0.61 ± 0.13	↓	*0.037*
GDF5 200 ng/ml	0.79 ± 0.12	0.50 ± 0.02	0.25 ± 0.17	0.66 ± 0.24	0.66 ± 0.12	↓	*0.018*
TIMP1							
No GF	1.00 ± 0.09	1.00 ± 0.14	1.00 ± 0.03	1.00 ± 0.07	1.00 ± 0.06		
GDF5 10 ng/ml	0.95 ± 0.16	1.02 ± 0.12	0.98 ± 0.06	0.68 ± 0.06	1.06 ± 0.20		*0.99*
GDF5 100 ng/ml	0.70 ± 0.18	0.59 ± 0.12	0.82 ± 0.28	0.66 ± 0.12	0.75 ± 0.13		*0.91*
GDF5 200 ng/ml	0.36 ± 0.08	0.60 ± 0.08	0.70 ± 0.09	0.63 ± 0.20	0.66 ± 0.07		*0.83*
TIMP3							
No GF	1.00 ± 0.42	1.00 ± 0.73	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.18		
GDF5 10 ng/ml	1.49 ± 0.64	0.98 ± 0.22	1.88 ± 0.29	1.21 ± 0.18	1.29 ± 0.33		0.23
GDF5 100 ng/ml	1.68 ± 0.53	4.20 ± 0.44	4.65 ± 1.31	1.78 ± 0.33	2.82 ± 0.15	↑	*<0.0001*
GDF5 200 ng/ml	2.78 ± 0.55	5.21 ± 1.91	3.50 ± 0.32	1.88 ± 0.98	2.83 ± 0.30	↑	*0.00014*

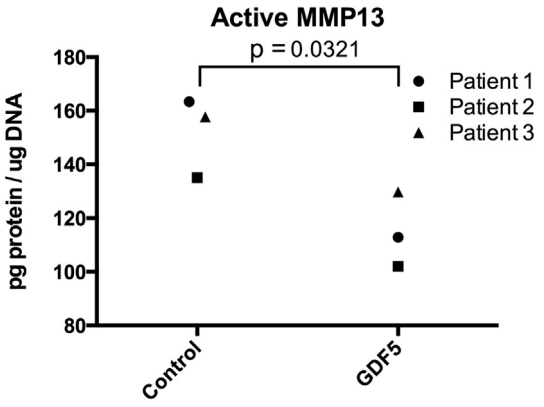

Fig. 1. Expression of active MMP13 protein was reduced by the chondrocytes when stimulated with 200 ng/ml GDF5 ($n = 3$, three pooled replicates). Results analyzed with ANOVA.

Sections (5 μm) were cut in the approximate center of the pellet and placed onto Super frost plus microscope slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized and stained with Alcian Blue van Gieson staining. Slides were examined under the microscope (Nikon Eclipse 90i, Nikon). Pictures were taken with a Nikon DXM1200F digital camera (Nikon) and the NIS Elements D software (Nikon).

DKK1, β-catenin and active MMP13 ELISAs

On day 18, 200 μl of fresh medium was added to the pellet cultures. On day 21 medium was collected and stored at −20°C until analysis. Medium (100 μl) from each culture was analyzed with the human DKK1 ELISA (R&D Systems) according to the manufacturers protocol. For the active MMP13 ELISA media from three pellets were pooled to a volume of 600 μl and concentrated to 250 μl with Amicon Ultra-4 Centrifugal Filter Unit, cut-off 3 kDa according to the manufacturers protocol (Millipore). Media (200 μl) from each sample were then analyzed with the active MMP13 ELISA (R&D Systems) according to the manufacturers protocol. For the total β-catenin, three pellets were pooled in 1.5 ml polypropylene tubes and stored at −80°C. ELISA kit lysis buffer (100 μl) was added to each tube with a 5 mm stainless steel bead (Qiagen). Samples were homogenized in a TissueLyser (Qiagen), 2 × 2 min, 25 Hz, and diluted with 50 μl of the ELISA kit lysis buffer and stored at −80°C until analysis. Total protein content was determined with a bicinchoninic acid protein quantification kit (Sigma). A human total β-catenin ELISA kit (R&D Systems) was used to analyze 100 μl of each sample, according to the manufacturers instructions. Protein concentrations in the samples were determined with a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

Ethical approval

Cartilage donations were approved by the ethical committee at The Sahlgrenska Academy, University of Gothenburg.

Statistics

The separate experiments in this study included 1–6 patients (n) per group as stated in figure texts, with three replicates per n unless otherwise stated. For qPCR analysis, data were normalized to non-stimulated control group. Results are displayed as mean for each patient ±95% confidence interval (CI) determined from three replicates. Differences between stimulations were analyzed by Student's t test or one-way analysis of variance (ANOVA) with the Tukey *post hoc* test and P-values considered significant at $P < 0.05$. To take intra-patient variability into account each replicate was used as a separate value. SPSS (IBM Corporation, Armonk, NY, USA) and GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) were used for statistical analyses. For the factorial DoE, multiple linear regression (MLR) using least square methodology

Table III
GDF5 stimulation shifted the chondrocytes into an anabolic state. Gene expression of *ACAN* and *SOX9* were increased upon GDF5 stimulation. Reduced expression of *COL10* was detected at the highest concentrations of GDF5 stimulation ($n = 5$, three replicates). Results analyzed with ANOVA, table showing mean ± 95% CI. GF = Growth factor. UD = Undetectable

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5		*P*-value
MMP13							
No GF	1.00 ± 0.03	1.00 ± 0.33	1.00 ± 0.02	1.00 ± 0.07	1.00 ± 0.08		
GDF5 10 ng/ml	1.03 ± 0.26	1.52 ± 0.31	4.54 ± 0.29	0.77 ± 0.21	4.07 ± 0.76		*0.95*
GDF5 100 ng/ml	3.57 ± 1.10	8.57 ± 2.21	5.72 ± 1.05	2.42 ± 0.34	16.36 ± 0.37	↑	*0.045*
GDF5 200 ng/ml	13.07 ± 3.07	11.01 ± 1.31	5.13 ± 0.88	5.11 ± 0.78	20.51 ± 1.14	↑	*0.00059*
SOX9							
No GF	1.00 ± 0.05	1.00 ± 0.28	1.00 ± 0.03	1.00 ± 0.09	1.00 ± 0.11		
GDF5 10 ng/ml	1.11 ± 0.22	1.01 ± 0.51	1.03 ± 0.06	1.00 ± 0.11	0.97 ± 0.11		*0.99*
GDF5 100 ng/ml	1.51 ± 0.33	1.56 ± 0.42	1.12 ± 0.16	1.77 ± 0.73	1.00 ± 0.09		*0.11*
GDF5 200 ng/ml	2.21 ± 0.22	1.57 ± 0.47	1.45 ± 0.56	1.81 ± 0.43	1.25 ± 0.11	↑	*0.0034*
COL10							
No GF	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.06		
GDF5 10 ng/ml	0.81 ± 0.12	0.75 ± 0.23	1.05 ± 0.12	0.37 ± 0.14	1.25 ± 0.10		*0.99*
GDF5 100 ng/ml	0.50 ± 0.09	0.46 ± 0.11	0.27 ± 0.05	UD ±	UD ±	↓	*0.014*
GDF5 200 ng/ml	0.43 ± 0.14	0.31 ± 0.15	0.25 ± 0.00	UD ±	UD ±	↓	*0.0060*
GDF5							
No GF	1.00 ± 0.18	1.00 ± 0.05	1.00 ± 0.13	1.00 ± 0.02	1.00 ± 0.06		
GDF5 10 ng/ml	1.09 ± 0.28	0.93 ± 0.12	0.95 ± 0.06	0.89 ± 0.07	0.82 ± 0.05		*0.99*
GDF5 100 ng/ml	0.86 ± 0.06	0.26 ± 0.01	0.39 ± 0.06	0.43 ± 0.03	0.34 ± 0.03	↓	*0.0070*
GDF5 200 ng/ml	0.44 ± 0.10	0.26 ± 0.09	0.30 ± 0.06	0.44 ± 0.16	0.24 ± 0.04	↓	*<0.0001*

was used for the analysis in Modde 8.0 and models considered significant at $P < 0.05$ and without a significant lack of fit at $P > 0.05$.

Results

GDF5 stimulation reduces expression of matrix degrading enzymes

Stimulating human chondrocyte pellets with GDF5 resulted in dose dependent decreases in gene expressions of the matrix degrading enzymes *MMP13* and *ADAMTS4*, with the strongest reduction at the highest GDF5 concentration, 200 ng/ml, for both *MMP13* (−33-fold, $P = 0.00038$) and *ADAMTS4* (−1.75-fold, $P = 0.018$), compared with control. *TIMP1* gene expression was unaffected by the GDF5 stimulation ($P = 0.67$) and *TIMP3* expression was increased (+3.2-fold, $P = 0.00014$) (Table II). The reduced gene expression levels of *MMP13* were reflected by reduced amounts of active MMP13 protein in the culture medium of pellets stimulated with 200 ng/ml GDF5 (−1.3-fold, $P = 0.032$) [Fig. 1], compared with control.

GDF5 stimulation shift human chondrocytes into an anabolic state

Expression of genes associated with chondrocyte differentiation and ECM production increased in a dose dependent manner in response to GDF5 stimulation, *ACAN* (+11-fold, $P = 0.00060$) and *SOX9* (+3.5-fold, $P = 0.0012$). GDF5 stimulation also reduced hypertrophic differentiation of the cells as *COL10* expression was downregulated at 100 ng/ml and 200 ng/ml GDF5 stimulation. The endogenous expression of GDF5 was decreased as a result of the stimulations (−3.0-fold, $P < 0.0001$) (Table III). After 21 days in culture there were significant dose dependent increases in total DNA content in pellets stimulated with GDF5 (+3.1-fold, $P = 0.00063$), compared with control. The GAG/DNA ratio, that reflects the matrix producing capacity of the cells, increased with GDF5 stimulation in a dose dependent manner (+4.3-fold, $P = 0.0015$), compared with control. Pellet size increased with GDF5 stimulation (+2.5-fold, $P < 0.0001$) compared with control (Table IV). Histology sections stained with Alcian Blue van Gieson confirmed the increase in proteoglycan rich ECM after GDF5 stimulation, as compared with control [Fig. 2].

Biological relevance of the in vitro system

To assess the biological relevance of the *in vitro* system used herein, expression levels of *GDF5*, *SOX9*, *ACAN*, *COL2A1*, and *MMP13* were compared to those in normal and OA cartilage biopsies [Fig. 3]. *ACAN* and *COL2A1* expressions were higher in the biopsies, *SOX9* and *MMP13* expressions were similar in the two systems, and *GDF5* expression was higher in the *in vitro* system.

GDF5 stimulation inhibits canonical Wnt signaling in human chondrocytes

GDF5 stimulation resulted in increased mRNA expressions of two inhibitors of canonical Wnt signaling, *DKK1* (+12-fold, $P = 0.00032$) and *FRZB* (+5.1-fold, $P = 0.017$) (Table V). An increase in DKK1 protein levels was measured in the medium of pellets cultured in the presence of 200 ng/ml GDF5 as compared with control (+4.0-fold, $P < 0.0001$) [Fig. 4(A)]. Protein quantification of β-catenin revealed reductions in total β-catenin in the GDF5 stimulated cells (−1.3-fold, $P = 0.00022$), indicating an inhibition of the canonical Wnt signaling pathway [Fig. 4(B)]. Inhibition of the signaling pathway was confirmed with qPCR, showing downregulation of the canonical Wnt pathway downstream genes *FOSL1* (−1.36-fold, $P = 0.016$) and *PPARD* (−1.33-fold, $P = 0.040$) [Fig. 4(C)–(D)].

GDF5 stimulation reduces Wnt signaling induced MMP13 expression

In order to investigate and validate the role of canonical Wnt signaling for the expression of matrix modulating genes in our culture system we activated the canonical Wnt pathway. Stimulation with the small molecule GSK3β inhibitor CHIR-99021

Table IV
Pellet size increased with increased GDF5 stimulation. The largest increase was seen at the highest GDF5 concentration ($n = 3$). Biochemical analysis of the pellets showed increased total amounts of DNA with increased GDF5 concentration. Amount of DNA per pellet at day 0 was 1.9 μg ($n = 5$, three replicates). The GAG/DNA ratio was also increased with increasing GDF5 concentration. Results analyzed with ANOVA, table showing mean ± 95% CI

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5		*P*-value
DNA							
No GF	2012 ± 119	333 ± 26	710 ± 163	3234 ± 390	1599 ± 763		
GDF5 10 ng/ml	3087 ± 958	401 ± 13	4555 ± 384	4994 ± 860	3939 ± 363		
GDF5 100 ng/ml	3526 ± 558	604 ± 104	5397 ± 57	5121 ± 2057	5605 ± 255	↑	*0.014*
GDF5 200 ng/ml	2957 ± 929	871 ± 273	6227 ± 592	7979 ± 2146	6296 ± 390	↑	*0.00062*
GAG/DNA							
No GF	1.00 ± 0.00	1.00 ± 0.00	0.87 ± 0.40	0.88 ± 0.26	1.01 ± 0.32		
GDF5 10 ng/ml	1.09 ± 0.81	0.91 ± 0.42	2.42 ± 0.32	1.63 ± 0.51	0.83 ± 0.35		
GDF5 100 ng/ml	3.26 ± 1.54	3.96 ± 0.26	3.07 ± 0.07	2.99 ± 0.48	3.30 ± 0.27	↑	*0.077*
GDF5 200 ng/ml	2.83 ± 1.51	5.20 ± 0.39	3.60 ± 0.11	3.45 ± 0.53	2.53 ± 0.10	↑	*0.015*
Pellet size							
No GF	1.00 ± 0.09	1.00 ± 0.04	0.99 ± 0.32				
GDF5 10 ng/ml	0.99 ± 0.09	0.95 ± 0.12	1.41 ± 0.35				
GDF5 100 ng/ml	1.44 ± 0.10	1.16 ± 0.09	1.79 ± 0.70			↑	0.0023
GDF5 200 ng/ml	2.93 ± 0.12	1.23 ± 0.11	2.34 ± 0.25			↑	0.00027

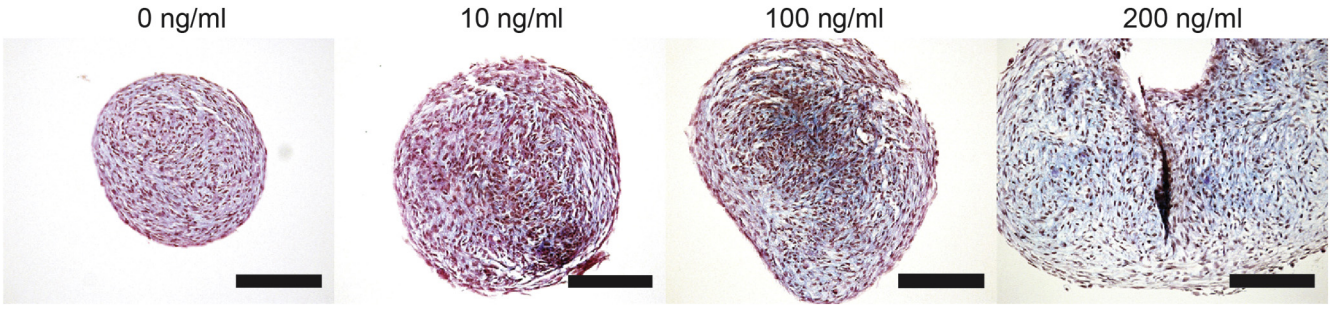

Fig. 2. Representative images of the histological pellet sections. Alcian Blue van Gieson staining showing increased proteoglycan expression with increased GDF5 concentration from 0 ng/ml (left) to 200 ng/ml (right), scale bar 200 μm ($n = 5$).

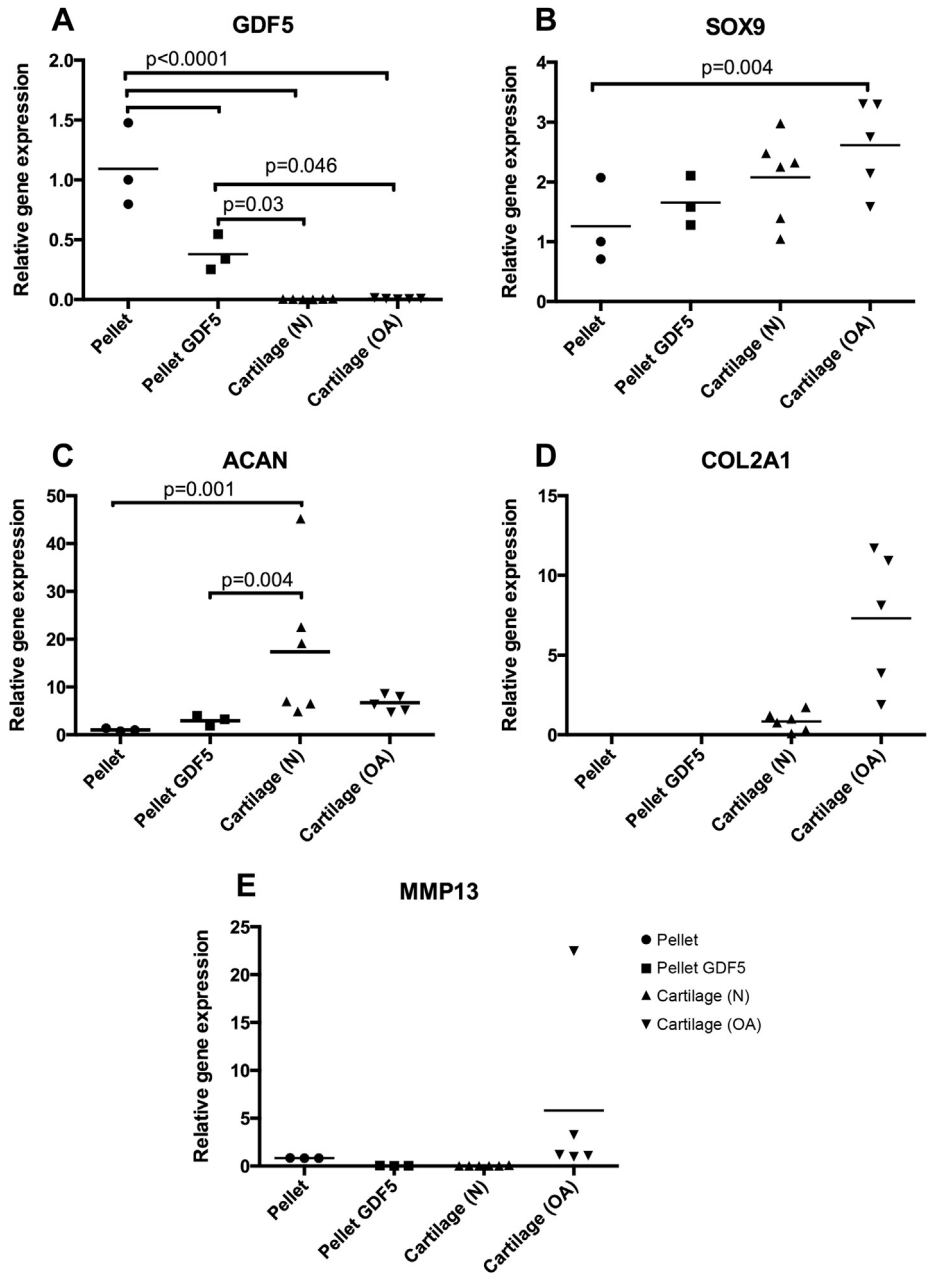

Fig. 3. Relative gene expression levels in non-stimulated pellets ($n = 3$, mean from three replicates each) and pellets ($n = 3$, mean from three replicates each) stimulated with 200 ng/ml GDF5 compared with expression levels in normal (N) ($n = 6$, no replicates) and OA ($n = 5$, no replicates) cartilage biopsies of selected markers A) *GDF5*, B) *SOX9*, C) *ACAN*, D) *COL2A1* and E) *MMP13*. Results analyzed with ANOVA, horizontal lines show mean.

Table V
GDF5 stimulation induced expression of Wnt inhibitors. Gene expressions of A) *DKK1* and B) *FRZB* increased in a dose dependent manner ($n = 5$, 3 replicates)

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5		*P*-value
DKK1							
No GF	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.15	1.00 ± 0.00		
GDF5 10 ng/ml	0.27 ± 0.00	1.00 ± 0.00	2.46 ± 0.14	2.30 ± 1.60	4.13 ± 1.42		*0.99*
GDF5 100 ng/ml	0.69 ± 0.23	1.19 ± 1.27	18.38 ± 8.29	9.21 ± 0.87	4.35 ± 1.73	↑	*0.012*
GDF5 200 ng/ml	3.34 ± 0.16	6.58 ± 0.06	24.47 ± 9.28	15.34 ± 6.20	10.02 ± 1.17	↑	*0.0024*
FRZB							
No GF	1.00 ± 0.12	1.00 ± 0.28	1.00 ± 0.03	1.00 ± 0.55	1.00 ± 0.13		
GDF5 10 ng/ml	1.20 ± 0.13	1.22 ± 0.66	1.93 ± 0.25	0.83 ± 0.12	1.94 ± 0.58		*0.85*
GDF5 100 ng/ml	3.62 ± 1.76	5.50 ± 2.49	2.72 ± 0.09	1.95 ± 0.29	4.57 ± 0.18		*0.11*
GDF5 200 ng/ml	6.22 ± 2.14	7.04 ± 3.07	3.43 ± 0.89	3.35 ± 1.02	4.96 ± 3.29	↑	*0.018*

increased expression of matrix degrading enzymes and decreased anabolic genes in the chondrocyte pellets, compared with control: *MMP13* (+5.1-fold, $P = 0.023$), *ADAMTS* ($P = 0.67$), *TIMP1* ($P = 0.09$), *TIMP3* (−2.0-fold, $P < 0.00001$), *ACAN* (−3.2-fold, $P < 0.00001$), *SOX9* (−0.51-fold, $P = 0.00094$) [Fig. 5(A)–(F)]. The active MMP13 ELISA showed that canonical Wnt pathway activation increases the MMP13 protein levels (+1.5-fold, $P = 0.047$), compared with control [Fig. 5(G)]. We further investigated if Wnt ligand activation could mediate the same matrix degrading effect. The chondrocytes were stimulated with recombinant Wnt3a ligand, which increased *MMP13* expression in the chondrocyte cultures (+1.87-fold, $P < 0.0001$). This increase was counteracted by GDF5 stimulation ($P < 0.0001$) [Fig. 6].

Inhibition of DKK1 restores GDF5 suppressed MMP13 expression

Finally we investigated if the decreased MMP13 expression in GDF5 stimulated chondrocyte pellets could be restored by blocking the DKK1 protein. Adding the DKK1 inhibitor WAY-262611 at 1 μM to chondrocyte pellet cultures stimulated with 200 ng/ml GDF5 fully restored the GDF5 suppressed *MMP13* gene expression [Fig. 7(A)]. The two-dimensional factorial design showed decreased

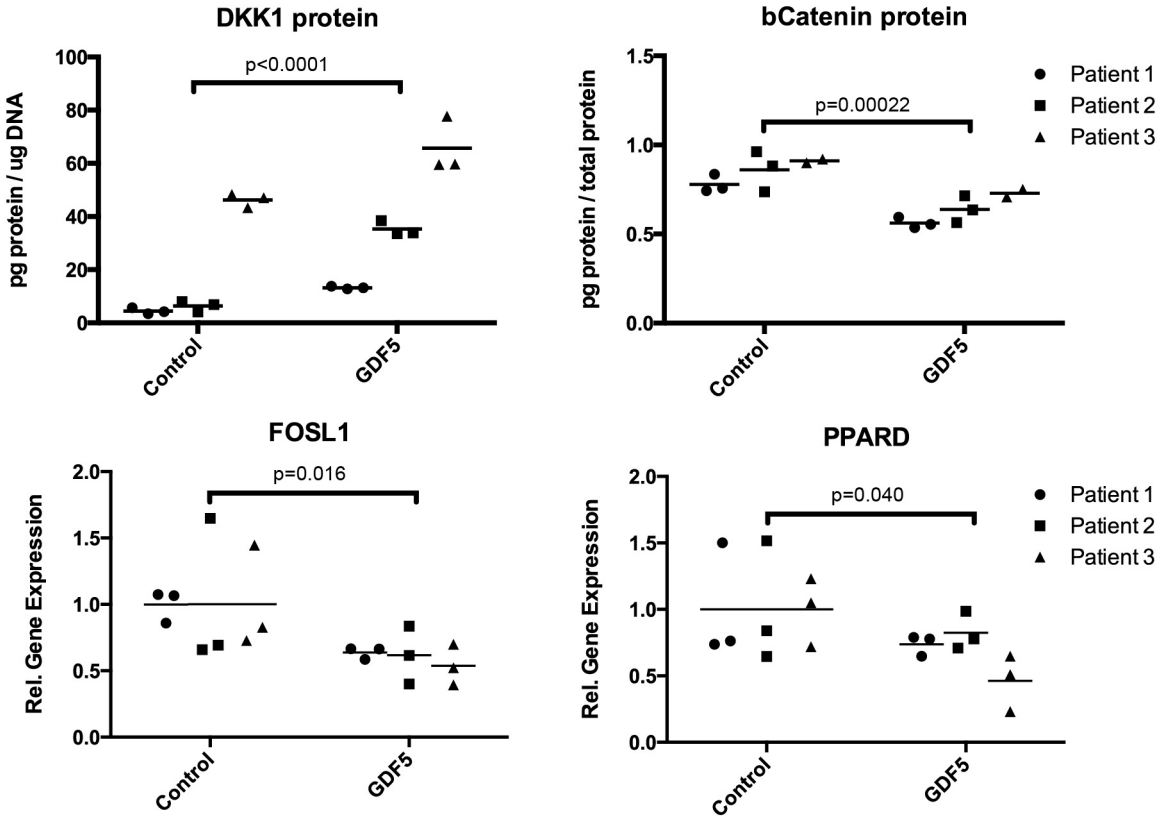

Fig. 4. Stimulating the pellets with 200 ng/ml GDF5 resulted in increased protein expression of A) DKK1 and reduced amounts of B) total β-catenin protein in the cells, indicating inhibited canonical Wnt signaling pathway. Reduced gene expressions of canonical Wnt downstream genes C) *FOSL1* and D) *PPARD* confirmed the inhibition of the canonical Wnt pathway ($n = 3$). Results analyzed with ANOVA, horizontal lines show mean.

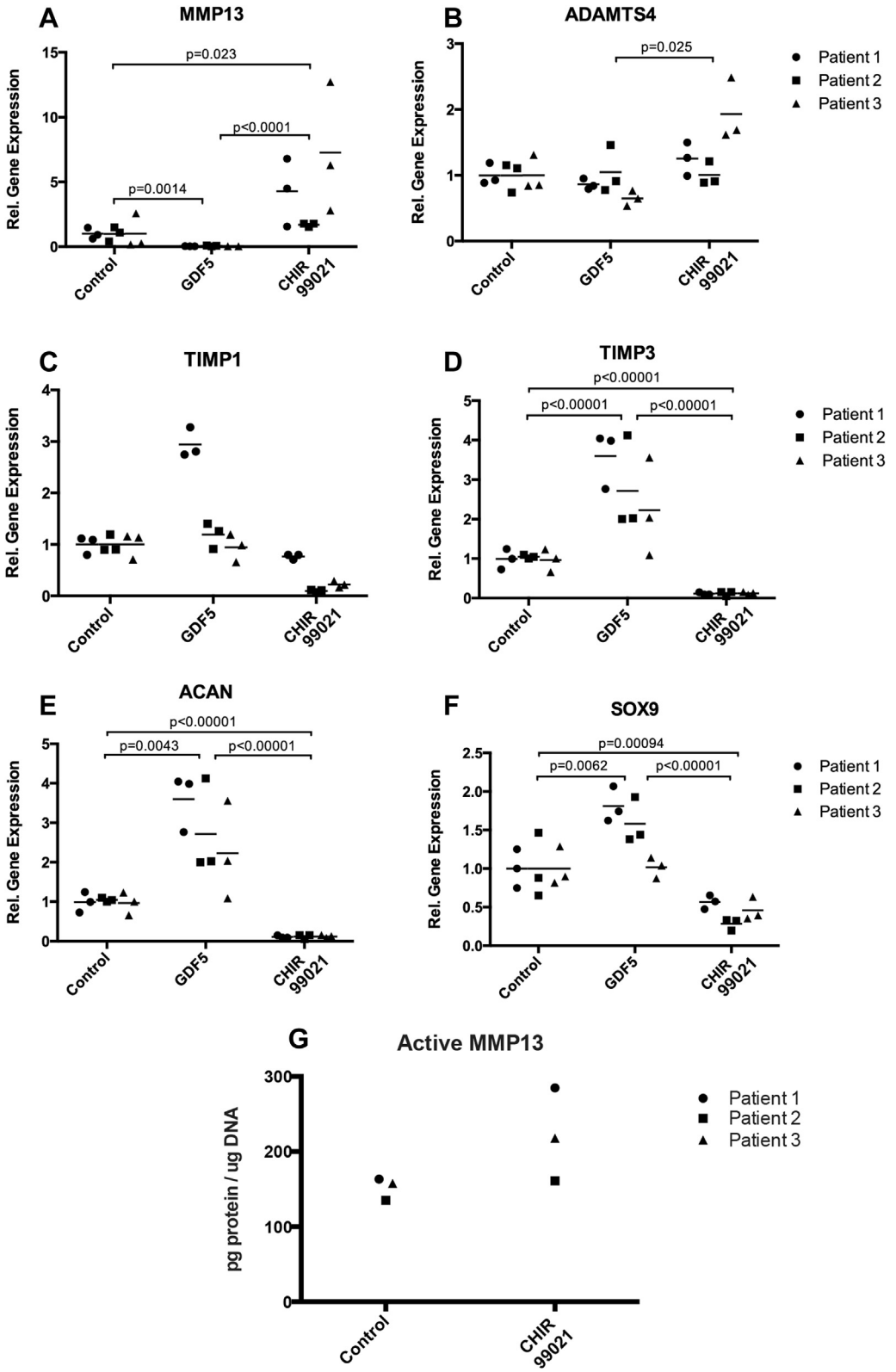

Fig. 5. Activating the canonical Wnt pathway by blocking GSK3β with the small molecule CHIR-99021 resulted in increased matrix degradation patterns in the cells with increased A) *MMP13* and B) *ADAMTS4* expressions, with unaffected C) *TIMP1* expression and decreased D) *TIMP3*, E) *ACAN* and F) *SOX9* expressions. Results analyzed with ANOVA, horizontal lines show mean ($n = 3$, three replicates). G) CHIR-99021 stimulation indicated increased active MMP13 protein expression in the cells ($n = 3$, three pooled replicates).

MMP13 gene expression and increased *ACAN* gene expression with increased concentration of GDF5. Simultaneous stimulations with the DKK1 inhibitor counteracted these effects in a dose dependent manner, $p_{\text{modelMMP13}} = 0.001$, $p_{\text{modelACAN}} = 0.002$ [Fig. 7(B) and (C)].

Discussion

As there is a lack of knowledge regarding the function of GDF5 in cartilage regeneration and disease, we investigated the effects of

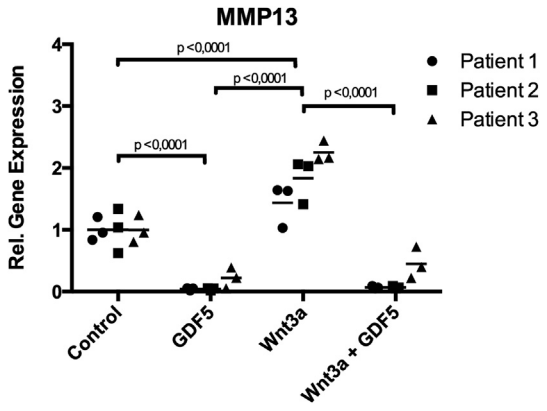

Fig. 6. Activating the canonical Wnt pathway with 50 ng/ml recombinant Wnt3a resulted in increased *MMP13* gene expression by the cells. This expression was counteracted by 200 ng/ml GDF5 stimulation ($n = 3$, three replicates). Results analyzed with ANOVA, horizontal lines show mean.

GDF5 stimulation in the pellet mass system, a frequently used *in vitro* system for studying chondrogenesis. We show that GDF5 stimulation has an inhibitory effect on the expression of catabolic genes, and a simultaneous stimulatory effect on the expression of anabolic genes in human articular chondrocytes. GDF5 stimulation increased the expressions of canonical Wnt inhibitors DKK1 and FRZB, and the canonical Wnt signaling was simultaneously inhibited. Finally, we show that this inhibition is DKK1-dependent and that the inhibition of the canonical Wnt pathway mediates the downregulation of MMP13 expression. Previous studies have shown that GDF5 has anabolic effects on chondrocytes[28,29,34] which was partly confirmed in the present study. However, GDF5 stimulation does not induce the same expressional levels *in vitro* of *ACAN* and *COL2A1* as the expression of these genes *in vivo*. Thus it could be argued that GDF5 does not have a strong anabolic effect when it comes to the expression of these genes in our model system. The increased levels of DNA after GDF5 stimulation compared to the start amount suggest a probable increased proliferation, confirming the results from Appel *et al.*[28], or possibly a combination of proliferation and reduced apoptosis. It should be noted that as the cells in the control pellets do not express *ACAN* or *COL2A1* to a high degree, these cells are reasonably dedifferentiated. This can be considered a major limitation to the study and the role for GDF5 in fully differentiated chondrocyte *in vivo* remains to be determined. However, the results have bearing on the regenerative and repair processes in cartilage in that the present study clearly shows a previously unknown biological mechanism, i.e., the connection between GDF5 stimulation and MMP13 expression through inhibition of Wnt signaling. The *MMP13* expression level in the analyzed cartilage is similar to the levels in the pellet system, which may suggest that the matrix protective effect of GDF5 stimulation could have physiological effects although the physiological relevance *in vivo* needs to be elucidated in future studies. Since the expression levels of *SOX9* and *MMP13* are similar in pellet cultures and biopsies we believe that the current model is appropriate to address the aim of the study.

GDF5 has previously been implemented in the OA disease in several publications. In the SNP analyses linking GDF5 to the development of OA, the SNP results in reduced promoter activity of

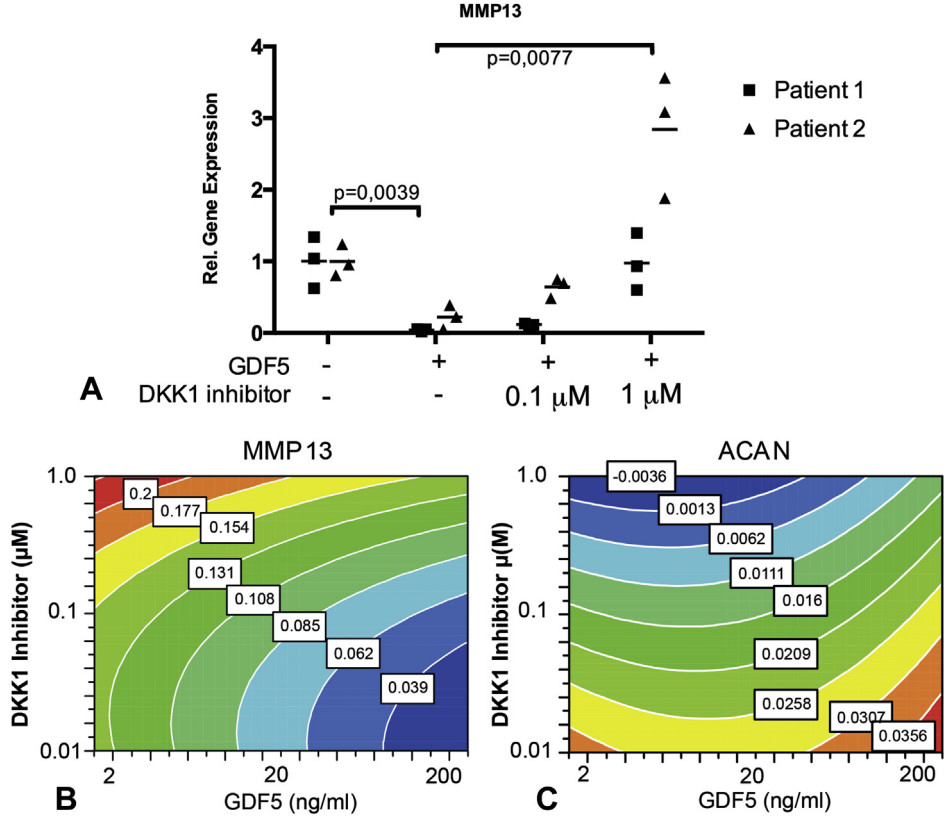

Fig. 7. A) Blocking DKK1 with the DKK1 inhibitor small molecule WAY-262611 restored the GDF5 reduced *MMP13* gene expression ($n = 2$, three replicates). Results analyzed with ANOVA, horizontal lines show mean. The response surfaces supported the linear results, with B) decreased *MMP13* gene expression with increased GDF5, and a counteracting effect by the DKK1 inhibitor. C) Analyzing the *ACAN* gene expression in the same system showed the opposite effects. Relative gene expression shown in white boxes in the graphs ($n = 1$, two replicates), data analyzed with MLR using least square methodology in computer software Modde 8.0. $p_{\text{modelMMP13}} = 0.001$, $p_{\text{modelACAN}} = 0.002$.

the GDF5 gene, reducing mRNA levels[26,27]. In this study, we show that the amounts of active MMP13 protein decreased after GDF5 stimulation and a simultaneous decrease in both *MMP13* and *ADAMTS4* mRNA levels. Hypothetically this effect could have been due to a decreased MMP13 release or increased breakdown of the protein but this is unlikely as we show a simultaneous effect on both mRNA and protein level. With the simultaneous increased, or unaffected, expression levels of their inhibitors (*TIMP3* and *TIMP1*), matrix degradation was likely reduced, suggesting that the presence of GDF5 could counteract degenerative processes in cartilage tissues. As the effect of GDF5 treatment was prominent *in vitro* although the cells had endogenous levels of GDF5 the effect may have physiological relevance *in vivo* since the expression was comparably low in cartilage biopsies. This needs to be elucidated in future animal studies. Our results suggest that reduced expressions of GDF5 caused by the rs143383 T/C SNP, could have impaired regulation of matrix degrading enzymes as a result, and even small stimulatory reductions may be important over time[27].

Our study shows that GDF5 not only affects expression of genes linked to cartilage ECM homeostasis, but also induces expression of Wnt inhibitor proteins DKK1 and FRZB in the chondrocytes. Both DKK1 and FRZB are implemented in the progression of OA. Loughlin *et al.*[15] showed that a SNP in the FRZB gene, resulting in diminished ability to disrupt Wnt signaling, increased susceptibility to hip OA. The DKK1 protein blocks canonical Wnt signaling and its role for the progression of OA is debated. Honsawek *et al.*[17] have shown that levels of DKK1 in plasma inversely correlates with severity of OA in human knees and Oh *et al.*[35] demonstrated that DKK1 inhibits Wnt mediated degradation of cartilage in mice. This is in agreement with the results from the present study, as blocking DKK1 with the inhibitor WAY-262611 abolished the GDF5 induced reductions in *MMP13* expression. The inhibitor also increased *MMP13* expression and decreased *ACAN* expression at the low, ultimately ineffective, doses of GDF5 as seen in the dose response surfaces in Fig. 7(B) and (C), likely depending on the blocking of the endogenous DKK1 expression.

In contrast it should be noted that Weng *et al.*[20,21] have shown in two studies that DKK1 stimulation induces chondrocyte apoptosis and cartilage destruction. This two faced role of DKK1 shows that its expression needs to be carefully controlled in the cartilage tissue.

In this study, following the increase of the inhibitors, the GDF5 stimulation led to reductions in β-catenin amounts in the cells and downregulation of downstream genes of the canonical Wnt pathway, indicating inhibition of the pathway. Similar to the role of DKK1, the canonical Wnt pathway is also two faced. Zhu *et al.*[36] described increased chondrocyte apoptosis after β-catenin inhibition and Yasuhara *et al.*[37] showed that Wnt signaling activation in mouse articular cartilage superficial zone cells stimulated proliferation and maintained a phenotypic expression of proteoglycan 4 and transcriptional regulator v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) *in vitro*. Several studies show the negative effects of active canonical Wnt signaling for cartilage formation and homeostasis with increased apoptosis and growth retardation in growth plate chondrocytes[6–8,38]. Miclea *et al.*[7] inhibited chondrogenesis by blocking GSK3β with the small molecule CHIR-99021, revealing that active canonical Wnt signaling increases cell death and MMP13 expression in chondrocytes, and that the resulting cartilage tissue is phenotypically similar to that of OA tissue. Active canonical Wnt signaling has also been shown to prevent early chondrogenesis and during endochondral bone formation it induces the maturation of hypertrophic chondrocytes with the expression of collagen X and MMP13, and it promotes bone formation[5,6,8]. Due to the contradictory reports of canonical Wnt signaling in relation to cartilage tissue homeostasis we investigated the effects of an active canonical Wnt signal in human chondrocytes in our culture system. Stimulation with the GSK3β inhibitor CHIR-99021 revealed increased matrix degradation. Activation of the pathway in the cells with recombinant Wnt3a similarly increased expression of MMP13. Interestingly, the effects of Wnt3a were counteracted by GDF5 stimulation. As shown in the present study this effect is likely mediated by DKK1 expression. Investigating which concentration levels of Wnt3a that GDF5 can counteract needs to be further elucidated in future studies in order to assess the physiological relevance.

Some members of the bone morphogenetic family, such as BMP2 and BMP4, are powerful inducers of bone formation in mesenchymal stem cells and in a study by Papathanasiou *et al.*[39], BMP2 was shown to activate the canonical Wnt pathway in human chondrocytes. This activation resulted in upregulation of *COL10* expression and induced hypertrophy. In comparison, no such effects were seen with the GDF5 stimulation in this study, as *COL10* expression was reduced by GDF5 stimulation.

In contrast to our results, underlining the dual role of the canonical Wnt signaling pathway, Ma *et al.*[40] showed in a short-term study that active canonical Wnt signaling in cartilage explants has inhibiting effects on *MMP13* expression on gene expression level. The differences to our study could be that our stimulation continued for a longer period of time and that our cells were retrieved from younger patients. Furthermore, in a study by Dell'Accio *et al.*[11], acute trauma was shown to increase levels of Wnt and Wnt associated proteins. The trauma also induced *MMP13* expression in human cartilage explants. An activation of the canonical Wnt pathway did, however, appear to be linked to a short-term upregulation of *COL2A1* and *ACAN*, indicating anabolic effects. The differences in results could stem from the different stimulatory time scales investigated, the difference in donor material and/or the differences in the way the pathway was stimulated.

Control of Wnt signaling is thus essential, as both up and downregulation of the pathway can affect cartilage negatively. Over longer time courses, canonical Wnt signaling seems to be harmful for cartilage tissue homeostasis.

In conclusion, the present study shows that GDF5 appears to have a role in the inhibition of canonical Wnt signaling and that it has effects on degenerative processes. The results may contribute to the knowledge about the molecular mechanisms responsible for the etiology of OA and could to some extent contribute to the understanding of the close link between the SNP in the GDF5 gene and the increased susceptibility to develop OA.

Author contribution

1. Conception and design: LE, JS, MB, AL. Collections and assembly of data: LE, JS. Analysis and interpretation of data: LE, JS, AL.
2. Drafting the article: LE, JS. Critical revision of the article for important intellectual content: LE, JS, MB, AL.
3. Final approval of the article: LE, JS, MB, AL. Obtaining of funding: AL.

Responsible authors for integrity of the study: L. Enochson (lars.enochson@gu.se) and A. Lindahl (anders.lindahl@clinchem.gu.se).

Competing interest

All authors state that they have no conflicts of interest.

Role of the funding source

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and interpretation of data, the writing of the manuscript or in the decision to submit the manuscript for publication.

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